Biosynthesis of Fatty Acids During Germination and Outgrowth of *Bacillus thuringiensis* Spores[1]

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Selective synthesis of branched- and normal-chain fatty acids occurred during germination and outgrowth of *Bacillus thuringiensis* spores. Iso-C_{13}, iso-C_{14}, and iso-C_{16} were the first fatty acids to appear upon germination, their formation occurring prior to protein synthesis. Afterwards, normal-C_{14}, normal-C_{16}, anteiso-C_{13}, and anteiso-C_{15} acids were synthesized maximally. During septum formation, anteiso-C_{17} acid appeared, and there was a resurgence in iso-C_{16} acid synthesis. Throughout outgrowth, iso-C_{15} and iso-C_{17} were metabolized uniformly, whereas the other acids exhibited peak synthesis.

Previously, we examined the kinetics of fatty acid synthesis during germination and outgrowth of *Bacillus thuringiensis* spores by analyzing [U-^{14}C]acetate and [2-^{3}H]glycerol incorporation into chloroform-methanol-extractable and trichloroacetic acid-precipitable lipids (6). The timing of fatty acid synthesis also was investigated relative to protein, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA) synthesis. RNA synthesis is initiated immediately upon germination, followed by rapid and extensive fatty acid synthesis which precedes protein, DNA, and triglyceride synthesis. The formation of fatty acids from acetate exhibits further developmental periodicity in which a large transient increase in fatty acid synthetic activity coincides with the approach of cell division.

In view of these relationships, we proceeded to investigate the timing of synthesis of the individual branched- and normal-chain fatty acids also. Differential fatty acid biosynthesis was correlated with morphological changes as well as with macromolecular synthesis.

MATERIALS AND METHODS

Organism and cultural conditions. *B. thuringiensis* NRRL B-4027 was obtained from the Agricultural Research Service Culture Collection, Peoria, Ill. Stock cultures were maintained on agar slants of MD medium (1), and spores for fatty acid and macromolecular analyses were obtained by growing the organism in a modified GYS medium (11) containing 0.2% $(NH_4)_2SO_4$, 0.2% yeast extract (Difco), and 0.05% K_2HPO_4 (adjusted to pH 7.3). After autoclaving, the following ingredients were added aseptically: glucose, 0.1%; $MgSO_4$, 0.02%; $CaCl_2 \cdot 2H_2O$, 0.008%; and $MnSO_4 \cdot H_2O$, 0.005%. The spores were harvested, washed once in distilled water and once in 0.05 M phosphate buffer (pH 7.2), and suspended in 100 ml of the phosphate buffer. Spores were heat-activated prior to germination by homogenizing for 2 min in a Potter-Elvehjem hand homogenizer, heat shocking for 30 min at 80 C, and homogenizing for an additional 2 min. The spores were germinated in the modified GYS medium to which L-alanine (100 μg/ml), adenosine (500 μg/ml), and ethylenediaminetetraacetic acid (EDTA, 25 μg/ml) had been added. The rate of germination, determined by decrease in optical density at 600 nm, was enhanced when EDTA was present.

Incorporation experiments. The techniques were similar to those previously published (8). Heat-activated spores were transferred at starting time (t_0) into flasks containing modified GYS medium, germinating agents, and the appropriate radioactive substrate. We employed L-[2,3-^{3}H]phenylalanine for indicating protein synthesis, [5,6-^{3}H]uridine for RNA, [6-^{3}H]thymidine for DNA, and [2-^{3}H]glycerol and [U-^{14}C]acetate for lipid synthesis. Whenever [6-^{3}H]thymidine was used, the medium was supplemented with 250 μg of 2-deoxyadenosine, uracil, cytosine, and guanosine (7) per ml to ensure incorporation of radioactive thymidine into DNA rather than into RNA. The flasks were rotated in a water bath at 28 C, and 1-ml samples were taken at intervals of either 2 or 5 min. Samples were added to 1 ml of 10% trichloroacetic acid containing carrier substrate (100 μg/ml) and were filtered on 25-mm membrane filters (0.45 μm, Millipore Corp.). The filters were washed four times with 5 ml of 5% trichloroacetic acid containing carrier substrate (50 μg ml), placed in 10 ml of scintillation fluid (10), and counted in a scintillation spectrometer.

Fatty acid analysis. Lipids were extracted from germinated and outgrown spores which had been harvested by centrifugation, washed three times in 0.85%

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NaCl, and stored in N_2 atmosphere at −20 C. The procedure for extracting lipids and for preparing methyl esters of fatty acids was described previously (1).

Methyl esters of the fatty acids were separated and determined on polar columns with an FSM model 810 dual-column chromatograph equipped with hydrogen flame detectors (1), and the amount of radioactivity in the methyl esters was analyzed with a gas chromatograph-liquid scintillation spectrometer system (2, 9). Computer programs (9) were utilized to process the radioactivity data. Relative specific activity of each ^{14}C-labeled ester was calculated by dividing the radioactivity percentage by the area percentage (3).

RESULTS

Macromolecular synthesis during germination and outgrowth. The importance of fatty acid synthesis to spore germination is best illustrated if the fatty acid data are examined within the framework of macromolecular synthesis in general and then correlated to known morphological stages determined by phase-contrast microscopy. All spores had completed germination by 15 min (t_{15}). They were subsequently observed to swell, elongate, undergo septum formation at t_{110}, and enter cell division at t_{130}.

Figure 1 illustrates the kinetics of [2-^{3}H]glycerol, [2,3-^{3}H]phenylalanine, [6-^{3}H]thymidine, [U-^{14}C]acetate, and [5,6-^{3}H]uridine incorporation into trichloroacetic acid-precipitable material. The data are in agreement with similar kinetic experiments done with non-crystal-forming aerobic sporeformers, in that RNA is the first macromolecule synthesized after germination. Interestingly, acetate was found to be rapidly incorporated starting at t_{20}, preceding detectable DNA, protein, and triglyceride synthesis. A substantial portion of this early acetate incorporation represented fatty acid synthesis. Throughout outgrowth, 40 to 50% of the acetate incorporated into trichloroacetic

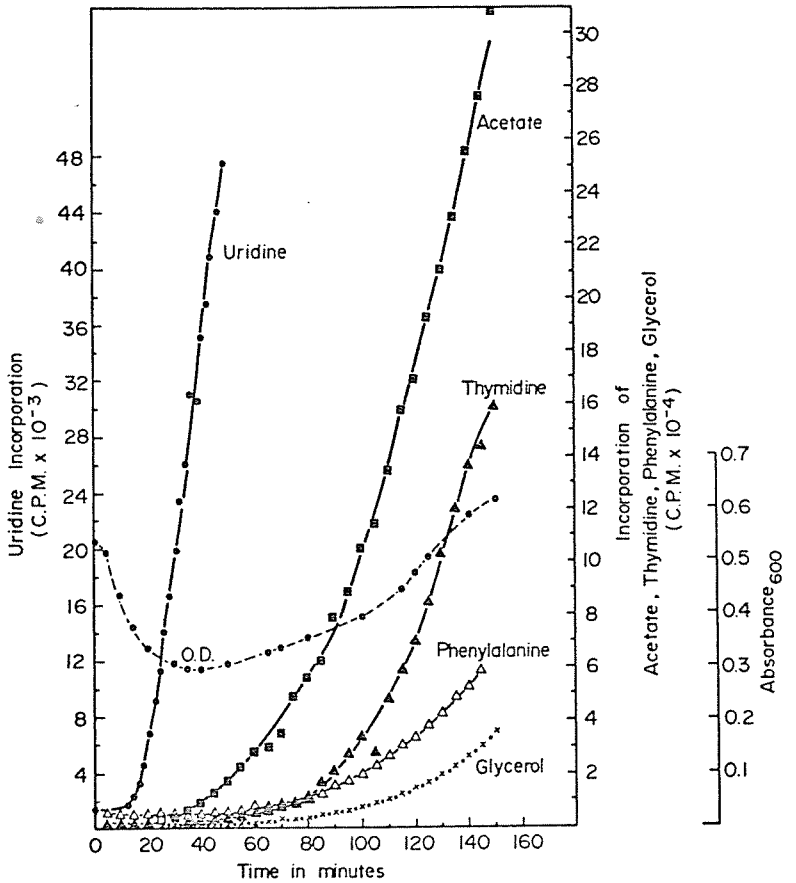

FIG. 1. *Kinetics of lipid, protein, ribonucleic acid, and deoxyribonucleic acid synthesis during outgrowth of Bacillus thuringiensis spores.*

acid-precipitable material was chloroform-methanol extractable. Differential synthesis of specific individual fatty acids was studied during four successive 30-min pulses covering the duration of germination and outgrowth.

Relative abundance of individual fatty acids in spores and vegetative cells. Many biochemical changes occur during spore germination, when the membrane fatty acid composition reverts from ratios characteristic of spore membrane to those typical of vegetatively growing cells. In Table 1, the percent relative abundance of individual fatty acids in spores (t_0) and vegetative cells (post-t_{120}) is compared. Iso-C_{13}, i-C_{15}, and a-C_{17} predominated in both. However, the levels of the branched-chain C_{13} and C_{15} homologues differed. Both the i-C_{13} and i-C_{15} isomers were far more prevalent than their corresponding isomers (a-C_{13} and a-C_{15}) in spores, whereas the values were approximately equal for vegetative cells. Significant differences were also observed between spores and vegetative cells in the relative abundance of the i-C_{11} and a-C_{17} fatty acids. For the even-numbered fatty acids, the iso homologues of C_{12}, C_{14}, and C_{16} were present in greater abundance than the normal isomers in both morphological stages. Overall, the iso homologues decreased during the transition from spores to vegetative cells, whereas the anteiso isomers increased; normal isomers remained constant.

Differential synthesis of fatty acids during germination and outgrowth. *B. thuringiensis* is typical of the gram-positive bacteria in that its membranes contain a high proportion of branched-chain fatty acids, as opposed to the normal- or straight-chain ones. Vegetative and spore membranes differ in their fatty acid composition, particularly in the ratio of iso to anteiso to normal isomers present (Table 1).

TABLE 1. *Percentage of fatty acids in the total lipids of spores and vegetative cells of Bacillus thuringiensis*

Fatty acid	Spores	Vegetative cells
i-C_{12}	1.29	1.25
n-C_{12}	0.44	0.42
i-C_{13}	11.82	7.48
a-C_{13}	2.20	5.96
i-C_{14}	9.81	5.94
n-C_{14}	4.38	3.45
i-C_{15}	27.36	18.92
a-C_{15}	10.13	19.39
i-C_{16}	7.76	7.66
n-C_{16}	5.72	7.42
i-C_{17}	6.14	7.12
a-C_{17}	11.73	14.70
Σ	iso-64.18 n-10.54 ante-24.06	iso-48.37 n-11.29 ante-40.05

TABLE 2. *Relative specific activity[a] of branched- and normal-chain fatty acids during germination and outgrowth of Bacillus thuringiensis spores*

Germination and outgrowth (pulse time in min)	Iso	Anteiso	Normal
0–30	99.9	0	0
30–60	35.4	32.5	32.2
60–90	39.5	20.4	40.1
90–120	44.3	27.3	25.9

[a] Relative percent incorporation of [1-^{14}C]acetate.

The relative amounts of each class of fatty acids synthesized were examined during four successive 30-min pulses following germination (Table 2). At no time did the proportions resemble the ratios of iso to anteiso to normal found for either intact spores or vegetative cells. Relatively large amounts of normal-chain acids were synthesized after the first 30 min; only iso fatty acids were formed during the first 30 min.

Iso fatty acids were synthesized to a greater extent than the homologous anteiso fatty acids, and the normal-chain acids were synthesized to a far greater extent than would be predicted from their final relative abundance in either spores or vegetative cells (Fig. 2). The ratio of iso to anteiso fatty acids synthesized in the last three pulses was approximately equivalent to that observed in vegetative cells.

Figure 3A-D shows the biosynthetic pattern of the even- and odd-chain iso, the odd-chain anteiso, and the even-chain normal fatty acids. These groupings were based on the biosynthetic origins of the fatty acids in question (5).

Iso-C_{13} was the only odd-chain iso isomer synthesized during t_{0-30} (Fig. 3A). Conversely, i-C_{15} and i-C_{17} were synthesized exclusively during the subsequent pulse periods. Iso-C_{15} attained maximal synthesis at t_{30-60} and then remained constant. Iso-C_{17} steadily increased from t_{30-60} through t_{90-120}, by which time septum formation was complete.

The only other fatty acids synthesized prior to t_{30} were i-C_{11} and i-C_{16} (Fig. 3B). [1-^{14}C]acetate was preferentially incorporated into i-C_{16} over i-C_{11} throughout cellular differentiation. The highest relative incorporation into i-C_{16} occurred during the t_{0-30} pulse; a rapid reduction of acetate carbons incorporated into i-C_{16} was evident during t_{30-60} with a marked recovery thereafter. Peak incorporation into i-C_{11} occurred during t_{30-60}. A similar pattern existed with i-C_{12}, only at a much lower level.

The patterns of a-C_{13} and a-C_{15} were similar

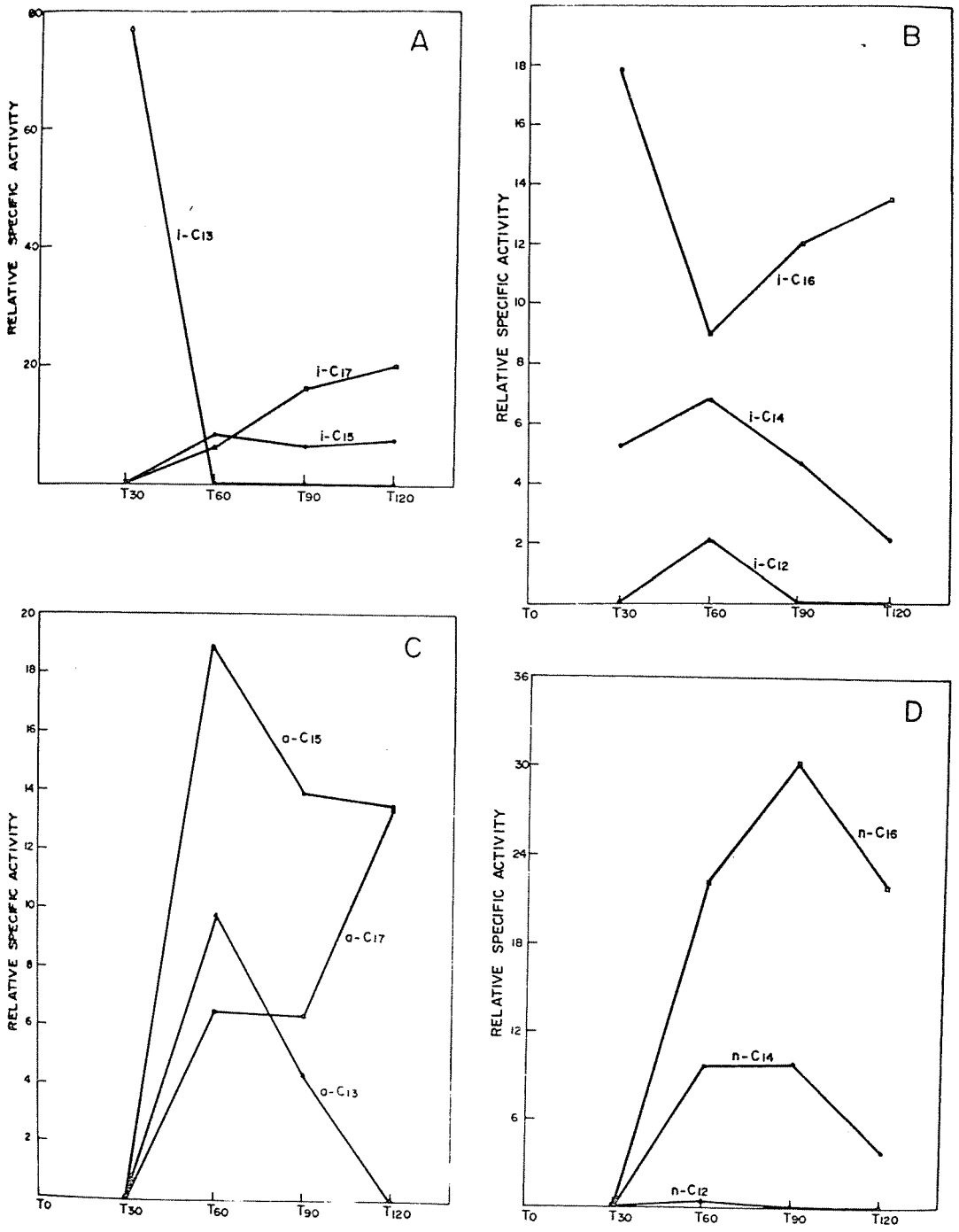

FIG. 3. *Differential synthesis of individual fatty acids during outgrowth of Bacillus thuringiensis spores. The radioactive fatty acids were prepared, extracted, and purified as described for Fig. 2. (A) Iso branched-chain fatty acids with odd number of carbon atoms. (B) Iso branched-chain fatty acids with even number of carbon atoms. (C) Anteiso branched-chain fatty acids with odd number of carbon atoms. (D) Normal straight-chain fatty acids with even number of carbon atoms.*

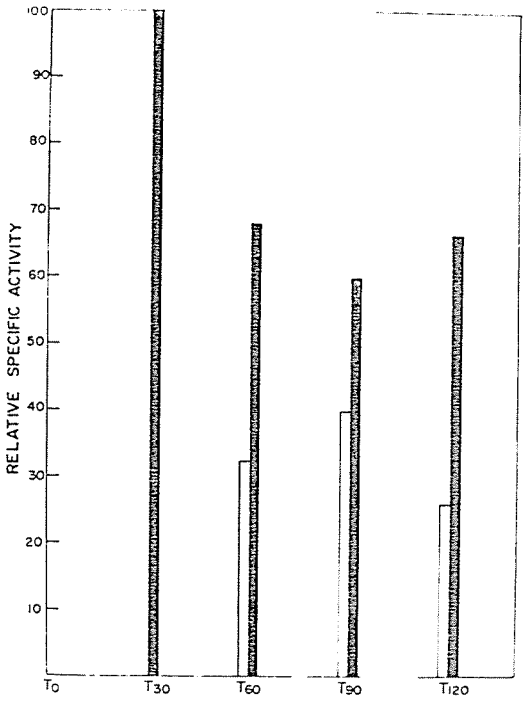

FIG. 2. *Differential synthesis of normal- and branched-chain fatty acids during outgrowth of Bacillus thuringiensis spores. Heat-activated spores (42 mg) were germinated at a concentration of 1.68 mg/ml in our standard GYS germination medium supplemented with 16 μg of sodium acetate/ml. Four pulse-label experiments were initiated by the addition of [1-^{14}C]acetate (1.2 μCi/ml) at t_0, t_{30}, t_{60}, and t_{90}. Each flask was harvested 30 min after isotope addition. The solid bars represent branched-chain fatty acid synthesis, and the open bars, normal-chain fatty acid synthesis.*

(Fig. 3C) in that maximal synthesis occurred at t_{30-60}, after which a-C_{13} synthesis dropped to zero. Although a-C_{15} was formed preferentially to the other odd-chain anteiso isomers, acetate incorporation into a-C_{17} became equal to that into a-C_{15} during the t_{90-120} pulse period.

Kinetic patterns of the even-chain normal fatty acids were similar (Fig. 3D). The differential rate of synthesis throughout the developmental period was n-C_{16} > n-C_{14} > n-C_{12}. Normal C_{16} was synthesized to the greatest extent at t_{60-90} and then decreased prior to cell division.

DISCUSSION

The differentiating cell has a marked capacity to synthesize new fatty acids, and this new synthesis is differential. There is a marked preference for which fatty acids are synthesized from acetate at a given time, and, in addition, the relative specific activity of each individual fatty acid varies substantially with time as the cell progresses through outgrowth. This differential synthesis implies that regulatory mechanisms control membrane fatty acid composition and that such changes in composition could determine developmental changes in membrane function. This latter possibility is rendered more likely by the high concentrations (up to 80%) of branched-chain fatty acids found in *B. thuringiensis* membranes (4).

Fatty acid synthesis during spore germination and outgrowth occurs in two distinct patterns, with a sharp division line existing at t_{30} (Fig. 3). This dichotomy may indicate that early fatty acid synthesis occurs in the absence of detectable de novo protein synthesis and thus is characteristic of the spore itself. This spore-specific fatty acid-synthesizing machinery would be present in the dormant spore and may reflect synthesis in the latter stages of spore formation. In this regard, the preponderant synthesis of i-C_{15} during t_{0-30} could be indicative of a selective function in either spore formation or spore germination.

The synthesis of normal-chain fatty acids commences subsequent to t_{30}; however, the relative specific activities of the aggregate normal-chain fatty acids (Table 2) far exceed their relative abundance in either the spore or vegetative stage (Table 1). These results indicate a disproportionate requirement for normal-chain fatty acids during outgrowth. The relative specific activities of the n-C_{14} and n-C_{16} fatty acids decrease during t_{90-120}, when the cells can be considered closer to having resumed vegetative growth (Fig. 3D). Also, the ratio of a-C_{15} to a-C_{17} (Fig. 3C) changes as the cells approach septum formation. The two fatty acids were synthesized in approximately equal amounts early in outgrowth (t_{30-60}), but thereafter a-C_{15} synthesis quickly dropped to zero while a-C_{17} synthesis increased dramatically. This change in fatty acid chain length may be related to septum formation and cell division.

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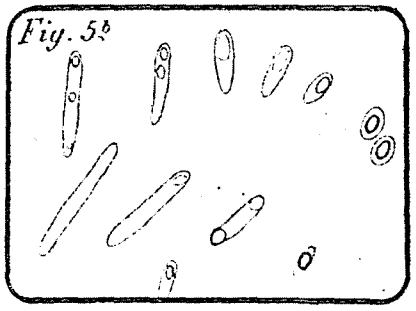
LITERATURE CITED

1. Bulla, L. A., Jr., G. A. Bennett, and O. L. Shotwell 1970 Physiology of sporeforming bacteria associated with insects. II. Lipids of vegetative cells. J. Bacteriol. 104:1246–1253.

2. Dutton, H. J. 1963. Monitoring gas chromotography for H^3- and C^{14}-labeled compounds by liquid scintillation counting, p. 147–152. *In* S. Rothchild (ed.), Advances in tracer methodology. Plenum Press, Inc., New York.
3. Dutton, H. J., and T. L. Mounts. 1966. Desaturation of fatty acids in seeds of higher plants. J. Lipid Res. **7**:221–225.
4. Kaneda, T. 1968. Fatty acids in the genus *Bacillus*. II. Similarity in the fatty acid compositions of *Bacillus thuringiensis*, *Bacillus anthracis*, and *Bacillus cereus*. J. Bacteriol. **95**:2210–2216.
5. Lennarz, W. J. 1966. Lipid metabolism in the bacteria. Adv. Lipid Res. **4**:175–225.
6. Nickerson, K. W., J. DePinto, and L. A. Bulla, Jr. 1975. Lipid metabolism during bacterial growth, sporulation, and germination: kinetics of fatty acid and macromolecular synthesis during spore germination and outgrowth of spores of *thuringiensis*. J. Bacteriol. **121**:227–233.
7. Rana, R. S., and H. O. Halvorson. 1972. Method for restricting incorporation of radioactivity from ^{3}H-thymidine into deoxyribonucleic acid only during outgrowth of spores of *Bacillus cereus* T. J. Bacteriol. **109**:599–605.
8. Rodenberg, S., W. Steinberg, J. Piper, K. W. Nickerson, J. Vary, R. Epstein, and H. O. Halvorson. 1968. Relationship between protein and ribonucleic acid synthesis during outgrowth of *Bacillus cereus*. J. Bacteriol. **96**:492–500.
9. Thomas, P. J., and H. J. Dutton. 1969. Improved resolution in high-sensitivity dual-label gas radiochromotography. Anal. Chem. **41**:657–660.
10. Winkler, H. H., and T. H. Wilson. 1966. The role of energy coupling in the transport of beta-galactosides by *Escherichia coli*. J. Biol. Chem. **241**:2200–2211.
11. Yousten, A. A., and M. H. Rogoff. 1969. Metabolism of *Bacillus thuringiensis* in relation to spore and crystal formation. J. Bacteriol. **100**:1229–1236.



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